

Supplementary Information

Exome sequencing in sporadic autism spectrum disorders identifies severe *de novo* mutations

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Supplementary Note

Clinical data. The 20 ASD trios sequenced were selected from the Simons Simplex Collection¹(SSC) (19) and the Study of Autism Genetics Exploration (SAGE) (1). The SSC is a Simons Foundation funded project including 12 collaborating sites across North America (see sfari.org). Each of the 12 data collection sites independently recruited families with ASD that had not previously participated in a genetics research study following approved human subjects research guidelines at each university. Participation in the collection was restricted to ASD simplex families in which both parents were willing to participate. Families in which the child with ASD had a relative (up to 3rd degree) with ASD or who had a sibling who showed ASD related symptoms, such as social challenges necessitating an individualized education program, were excluded. Inclusion criteria required that children with meet ASD diagnosis standards on the Autism Diagnostic Observation Schedule² (ADOS) and the Collaborative Programs for Excellence in Autism (CPEA) criteria for the Autism Diagnostic Interview, Revised³ (ADI-R). CPEA criteria require the child to score within 2 points of the cut-off on social or communication domains or within 1 point on both, with no requirement for the repetitive behavior or onset domains. Children also need to meet a nonverbal IQ estimate cut-off of 35. Additionally, children with significant hearing, vision or motor problems, significant birth complications (e.g. extended NICU stay), or having been diagnosed with ASD related disorders, such as Fragile X, were excluded.

Participation in the SSC for the children with ASD included a diagnostic evaluation, cognitive and adaptive assessment, comprehensive examination of medical and diagnostic history, and assessment of social and communicative abilities. Height, weight, head

circumference, and DNA via blood sample were collected from all participating family members and social communicative abilities were also assessed in the parents and siblings of the children with ASD. Data collection, entry, and validation methods were standardized across sites to ensure reliability of sample collection. Each institution applied for and received approval from the institution's human subjects division. As required by each local institutional review board, all subjects provided consent to participate in the collection.

Recruited families participating in the SAGE study range in age from 24 months old to adulthood and meet diagnostic criteria for ASD with no other factors likely to contribute to the etiology (such as very low birth weight, other genetic conditions) or diagnostic criteria for non-ASD developmental delay. Families are recruited from clinic patients undergoing a diagnostic evaluation for autism at Seattle Children's Autism Center. Following recruitment and enrollment, DNA samples and family history information are collected and diagnoses are confirmed through record review. As reported by parent informant, ~75% are simplex families. For those families with identified pathogenic event, a comprehensive phenotypic workup is conducted that includes: medical and family history; treatment history; diagnostic workup; cognitive testing; language, adaptive, and motor skills assessment; 2D photos of face & hands; and completion of parent questionnaires re: broader autism phenotype, general psychological functioning, aberrant behaviors, social symptoms, and demographic information. The SAGE study received approval from the Seattle Children's Human Subjects Division and all subjects provided consent to participate in the collection.

DNA samples were de-identified prior to distribution. The use of non-identifiable biological materials in this study was deemed not human subjects research by the University of Washington Human Subjects Division.

Detailed Clinical Information. NOTE: Standardized instruments included (mean 100, standard deviation is 15): cognitive: Differential Ability Scales-2nd edition, receptive language: Peabody Picture Vocabulary Test-4th edition, and adaptive skills: Vineland Adaptive Behavior Scales-2nd edition. Calibrated Severity Score is on 1-10 scale (>4 clinical; 10 most impaired). Abbreviations: Autism Diagnostic Interview (ADI); Autism Diagnostic Observation Schedule (ADOS); Intellectual Quotient (IQ).

ID #: SSC 12817

Mutation: *FOXP1* frameshift

Demographics: 9:5 male, White, non-Hispanic.

Family and Developmental History: No family history of ASD. Proband is 3rd of 3 siblings (1 older brother, 1 older sister). Paternal side: No significant psychiatric history. Maternal side: no significant psychiatric history. Pregnancy and birth: proband was 3rd pregnancy and delivery. Vaginal delivery at 41 weeks. Augmented labor. No anesthesia. No pregnancy or birth complications. No history of Rubella, Valproate use, Infections, Trauma, or use of Artificial Reproductive Technology.

Medical History: Likelihood of non-febrile seizures in proband (and paternal cousin).

Paternal/Maternal age in years at conception: 40/35.

Cognitive: FullScale IQ: 36 (<1st percentile); Nonverbal IQ: 34 (<1st percentile); Verbal IQ: 37 (<1st percentile); single word use.

Receptive Language: <1st percentile.

Adaptive Behavior: Daily Living Skills: 2nd percentile; Communication: 1st percentile; Social-Emotional: 2nd percentile; Overall: 1st percentile.

Diagnostic: Meets research criteria for Autistic Disorder: (ADI +; ADOS +; clinical diagnosis +). Calibrated Severity Score = 8. Evidence of onset prior to 3 years of age. Regression (word loss). Language delay (phrases delayed).

Aberrant Behaviors: Elevated for lethargy, hyperactivity, and inappropriate speech.

ID #: SSC 12681

Mutation: *GRIN2B* 3' splice

Demographics: 6:6 female, White, non-Hispanic.

Family and Developmental History: No family history of ASD. Proband is 3rd of 3 siblings (2 older sisters). Paternal side: No significant psychiatric history. Maternal side: no significant psychiatric history. Pregnancy and birth: proband was 3rd pregnancy and delivery. Upper respiratory infection and allergies reported during first trimester. Vaginal delivery at 41 weeks. Augmented labor. No anesthesia. Nuchal cord. No other pregnancy or birth complications. No history of Rubella, Valproate use, Trauma, or use of Artificial Reproductive Technology.

Medical History: Nothing of note.

Paternal/Maternal age in years at conception: 33/31.

Cognitive: FullScale IQ: 63 (1st percentile); Nonverbal IQ: 65 (1st percentile); Verbal IQ: 69 (2nd percentile); fluent language use.

Receptive Language: 21st percentile.

Adaptive Behavior: Daily Living Skills: 13th percentile; Communication: 5th percentile; Social-Emotional: 18th percentile; Motor: 7th percentile; Overall: 6th percentile.

Fine Motor Ability: Greater than 2 standard deviations below the mean.

Diagnostic: Meets research criteria for Autistic Disorder: (ADI +; ADOS +; clinical diagnosis +). Calibrated Severity Score = 9. Evidence of onset prior to 3 years of age. Possible regression.

Language delay (single word & phrases delayed).

Aberrant Behaviors: Elevated for hyperactivity.

ID #: SSC 12499

Mutation: *SCN1A* PRO1894LEU

Demographics: 6:11 male, White, non-Hispanic.

Family and Developmental History: No family history of ASD. Proband is 2nd of 2 siblings (1 older brother). Paternal side: cerebral palsy (pat cousin). Maternal side: migraines (mother, mat grandparent), lymphangioleiomyomatosis (lavi) (mat aunt/uncle). No significant psychiatric history. Pregnancy and birth: proband was 2nd pregnancy and delivery. Upper respiratory infection in trimester 2. Vaginal delivery at 40 weeks. No anesthesia. No pregnancy or birth complications. No history of Rubella, Valproate use, Trauma, or use of Artificial Reproductive Technology.

Medical History: Definite non-febrile seizures, dx of epilepsy.

Paternal/Maternal age in years at conception: 45/31.

Cognitive: FullScale IQ: 60 (<1st percentile); Nonverbal IQ: 57 (<1st percentile); Verbal IQ: 56 (<1st percentile); phrase speech use.

Receptive Language: 13th percentile.

Adaptive Behavior: Daily Living Skills: 8th percentile; Communication: 13th percentile; Social-Emotional: 6th percentile; Motor: 10th percentile; Overall: 76th percentile.

Diagnostic: Meets research criteria for Autistic Disorder: (ADI +; ADOS +; clinical diagnosis +). Calibrated Severity Score = 8. Evidence of onset prior to 3 years of age. Possible regression.

Language delay (single word & phrases delayed).

Aberrant Behaviors: No elevations.

ID #: SSC 11666.p1

Mutation: *LAMC3* ASP339GLY

Demographics: 7:9 male; White, non-Hispanic.

Family and Developmental History: no family history of ASD. Proband is 2nd of 2 children (1 older brother). Maternal side: migraines (mother); speech delay requiring therapy (maternal cousin); asthma (maternal cousin). Paternal side: eating disorder (paternal aunt, paternal grandmother), eczema (paternal grandparent). Pregnancy and birth: proband was 3rd pregnancy and 2nd delivery. Vaginal delivery at 40 weeks. Induced labor (failure to progress) via use of IV oxytocin. Anesthesia used. No pregnancy or birth complications. No history of Rubella, Valproate use, Infections, Trauma, or use of Artificial Reproductive Technology.

Medical History: Otitis Media at age 4 (treated). No other medical complications.

Paternal/Maternal age in years at conception: 32 years/30 years.

Cognitive: FullScale IQ: 49 (<1st percentile); Nonverbal IQ: 64 (1st percentile); Verbal IQ: 51 (<1st percentile); fluent language use.

Receptive Language: <1st percentile.

Adaptive Behavior: Daily Living Skills: 1st percentile; Communication: 2nd percentile; Social-Emotional: 2nd percentile; Overall: 1st percentile.

Fine Motor Ability: Greater than 2 standard deviations below the mean.

Diagnostic: Meets research criteria for Autistic Disorder: (ADI +; ADOS +; clinical diagnosis +). Calibrated Severity Score = 10. Evidence of onset prior to 3 years of age. No regression.

Aberrant Behaviors: Elevated for Hyperactivity and Irritability; Elevated for Stereotyped and Restricted behaviors.

Functional characterization of FOXP1 mutation. To assess whether the *FOXP1* frameshift mutation is targeted by nonsense-mediated decay (NMD), we grew two parallel cultures of immortalized lymphoblasts derived from the family, one of which was chemically treated to inhibit NMD. In the untreated sample we see low expression of the *FOXP1* insertion allele, while in the treated sample we see roughly equal levels of the frameshift mutation and the normal allele, suggesting that most of the mutated transcript is degraded (**Supplementary Fig. 5a**). Residual mutated p.A339SfxX4 transcripts are predicted to yield a C-terminally truncated protein that lacks the characteristic forkhead-box DNA-binding domain. We analyzed the functional properties of this FOXP1 mutant protein (FOXP1mut), making comparisons to wild-type FOXP1, FOXP2, and previously studied FOXP2 variants, using transfected HEK293T cell lines (**Supplementary Fig. 5b-d**). FOXP1mut displays aberrant localization to the cytoplasm as opposed to the nucleus—similar to results obtained with FOXP2 mutations⁴. This disruption of nuclear targeting is most likely due to the loss of nuclear localization signals flanking the DNA-binding domain⁴. In contrast to a truncated version of FOXP2 previously associated with verbal dyspraxia^{4,5}, the FOXP1mut product appears stable.

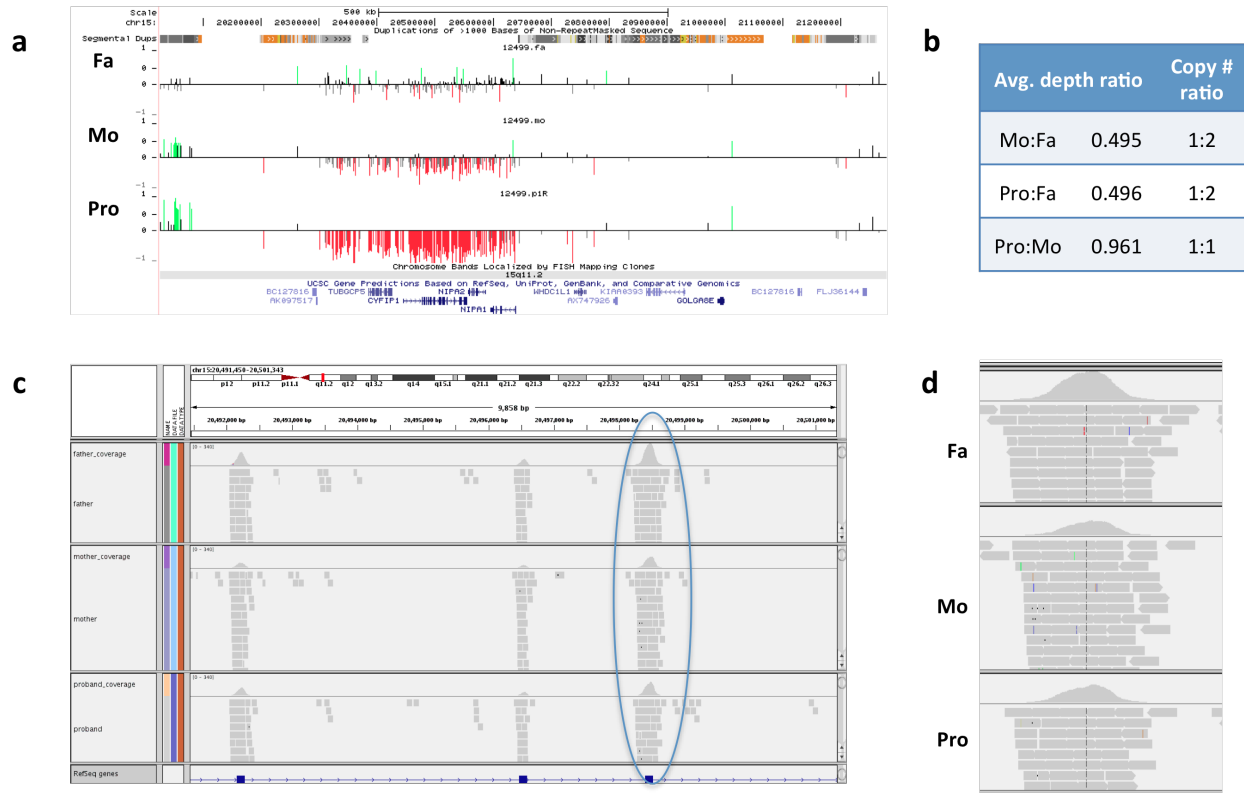
HEK293T cell-based assays indicated that, as for FOXP2⁶, increased levels of wild-type FOXP1 yield significantly reduced expression of *CNTNAP2* ($p=0.0005$) (**Supplementary Fig. 5d**). Intriguingly, in these experiments expression of the FOXP1mut protein was instead associated with a significant three-fold increase in *CNTNAP2* expression relative to control cells

($p=0.0056$). These data suggest that the aberrant FOXP1mut protein can interfere with the action of endogenous FOXP transcription factors present in the HEK293T cells⁴, causing a further misregulation of *CNTNAP2* expression. Overall, we hypothesize that reduced dosage of wild-type FOXP1 transcripts (due to NMD of mutant transcripts), combined with dysfunction of FOXP1mut proteins that escape this process, may yield overexpression of CNTNAP2 proteins, amplifying any deleterious effects of H275A in the proband. Negligible expression of *CNTNAP2* in our only available tissue (immortalized lymphoblasts) precludes direct testing of this hypothesis⁷.

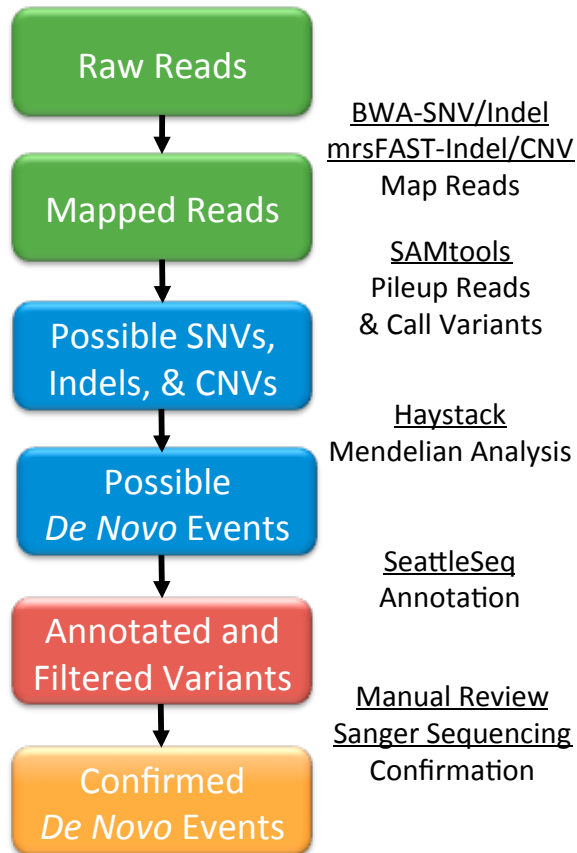
Comparison to Vissers *et al.* Vissers and colleagues reported a similar study on sporadic moderate to severe ID using Agilent/SOLiD technologies⁸. In contrast to our study, they reported putatively causative events (one of which was inherited) in 6/10 families suggesting that coding *de novo* events of major effect may be more prevalent in ID than ASD. Using an analogous approach, they reported that the predicted disease-associated events occurred at highly conserved sites and had disruptive Grantham scores. While the two approaches are similar, we did observe subtle differences in the mutation rates and characteristics. For example, although we both report ~1 coding *de novo* event per trio and sufficient coverage of ~90% of target, their set was larger (37 Mb versus 26 Mb). Based on our observed rate we would expect ~1.3 events per trio from a 37 Mb exome capture (versus the ~1.0 events per trio reported in their study). Based on their reported rate, we would expect ~0.7 events per trio from a 26 Mb capture (versus the ~0.9 events per trio observed in our study). In addition, 9/16 (56%) of the *de novo* coding substitutions we observed were nonsynonymous, while they reported 8/9 (89%) were nonsynonymous. We also observed markedly different transition/transversion ratios: 18:2 in this study versus 6:3 in the Vissers *et al.* study. However, none of these differences are statistically significant, and given the

relatively small number of observations at this time, it is unknown whether they reflect any true differences in either capture/sequencing/analysis methodology or in ID versus ASD patient populations.

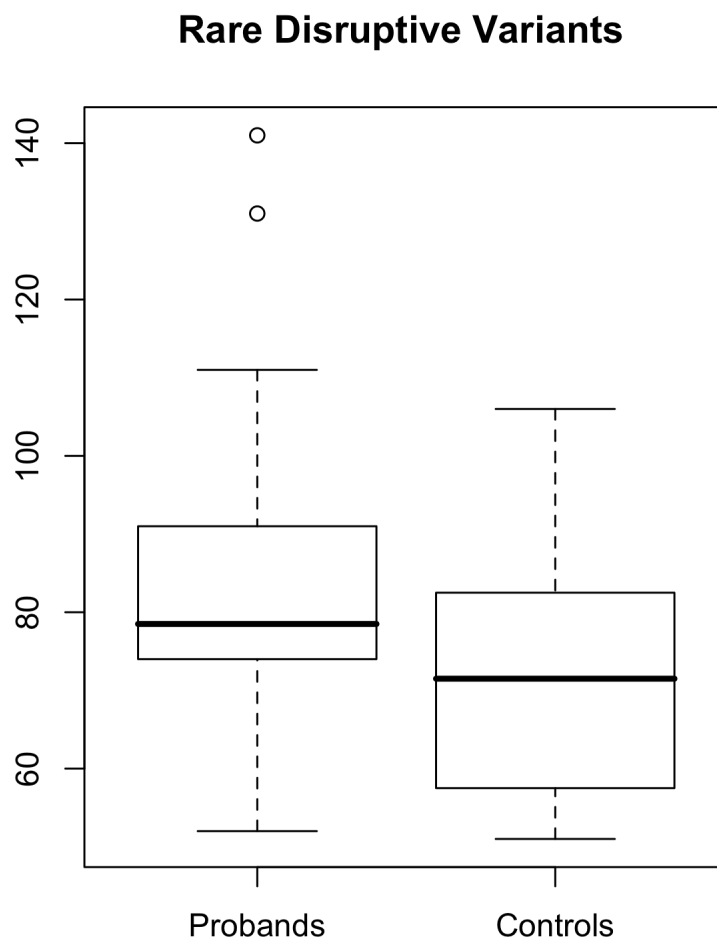
Description of other conserved/damaging variants. The remaining *de novo* mutation sites were not considered to be strong candidates for involvement in ASD, although it is entirely possible that sequencing of additional ASD cases may further implicate one or more of them. Missense mutations affecting conserved nucleotides and also predicted to be damaging based on Grantham scores occurred in *SYNE1*, *SLC30A5*, *TLK2*, and *RBM3*. *SYNE1* (p.Y282C, CCDS5236.1) is a spectrin repeat containing protein expressed in skeletal and smooth muscle, an extremely large transcript, and previously associated with spinocerebellar ataxia, autosomal recessive 8, and emery-dreifuss muscular dystrophy 4. *SLC30A5* (p.S561R, CCDS3996.1) is a zinc solute carrier that functions in the pancreas. *TLK2* (p.S595L, CCDS11633.1) is a serine/threonine kinases potentially involved in the regulation of chromatin assembly. *RBMS3* (p.T383M, CCDS33724.1) is an RNA-binding protein and potential regulator of hepatic stellate cells. The three remaining missense variants affect sites that are neither highly conserved nor predicted to be disruptive from the Grantham score. *TGM3* (p.V144I, CCDS33435.1) is a transglutaminase involved in hair follicle development. *GPR139* (p.S151G, CCDS32398.1) is a G-coupled receptor, which is expressed in the brain. *XIRP1* (p.V483M, CCDS2683.1) is the Xin actin-binding repeat-containing protein 1, associated with cardiomyopathy, and likely functions to protect actin filaments from depolymerization. The only synonymous and untranslated region (UTR) sites that occur at conserved nucleotides are at *ARHGAP15* and *MYO1A*, respectively. *ARHGAP15* is an RHO GTPase-activating protein, weakly expressed in brain. *MYO1A* is a myosin superfamily gene, associated with autosomal dominant deafness.



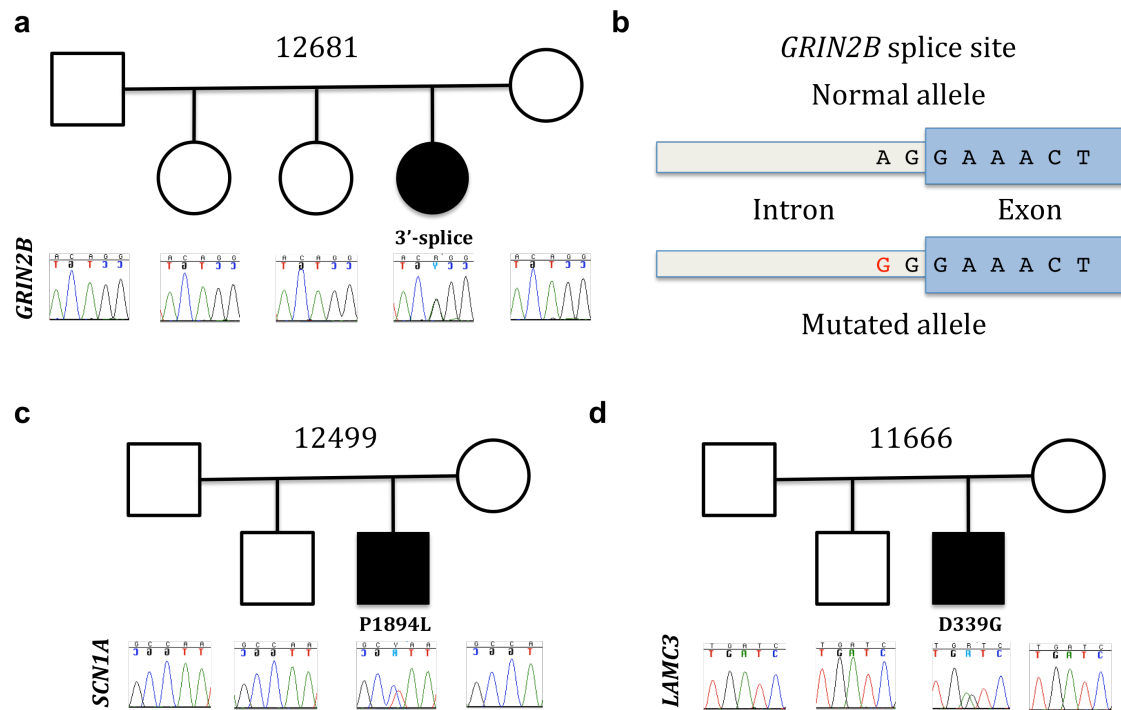
Supplementary Figure 1 Maternally inherited large CNV at 15q11.2 in family 12499. **a**, Genome browser diagram showing raw array CGH results indicating that the mother and proband are heterozygous deletion carriers. The deletion spans from approximately chr15:20,300,028-20,647,960, including *TUBGCP5*, *CYFIP1*, *NIPA2*, and *NIPA1*. Red indicates significantly deviated probes. **b**, Average exome read depth across the interval shows the expected relative copy numbers of the trio. **c**, IGV browser⁹ view shows drop in read depth across multiple exons. **d**, Expanded view of exon highlighted in **c**.



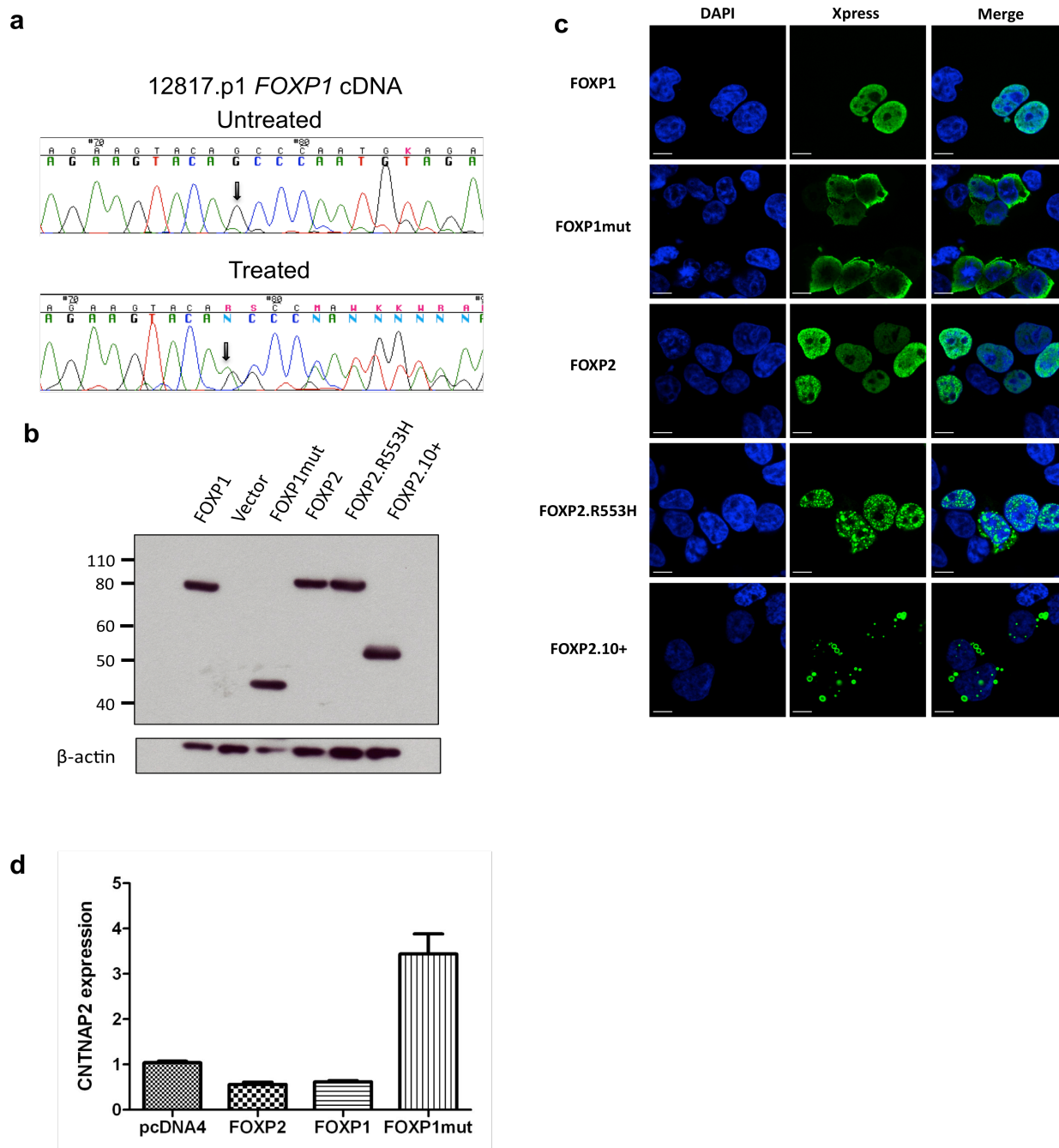
Supplementary Figure 2 Flow diagram of sequence analysis pipeline. First, raw reads are mapped to the reference genome using with *BWA* for SNV/small indel detection and *mrsFAST* for indel/CNV detection. Second, discordant read pairs and duplicates are removed, followed by genotype calling using *SAMtools*. Third, high quality variants are then run through a custom pipeline, *Haystack*, to evaluate their inheritance. Fourth, variant positions flagged as potentially *de novo* are then filtered against other sequenced exomes to remove systematic artifacts and annotated using the SeattleSeq server. Lastly, novel variants (e.g. not called in dbSNP, the 1000 Genomes Project Pilot, or 1490 other exomes) are then visually inspected to remove variants with >10% variant alleles in one or both parents and the remainder subjected to bi-directional Sanger sequencing.



Supplementary Figure 3 Boxplots showing number of “private” rare protein disruptive variants identified in the ASD proband and HapMap controls.



Supplementary Figure 4 SNV *de novo* mutation events that are potentially causative. **a**, Pedigree showing chromatogram traces surrounding *GRIN2B* 3' splice mutation. **b**, Diagram showing the affected *GRIN2B* intron-exon boundary. The A->G mutation results in loss of the canonical 3' AG splice site. **c**, Pedigree showing chromatogram traces surrounding *SCN1A* p.P1894L missense mutation. **d**, Pedigree showing chromatogram traces surrounding *LAMC3* p.D339G missense mutation.



Supplementary Figure 5 Functional characterization of *FOXP1* *de novo* mutation. **a**, Sanger traces of PCR amplified cDNA from 12817.p1 lymphoblasts. Untreated cells show low levels of the mutant allele p.A339SfsX4. Cells treated to inhibit NMD show approximately equal levels of

the normal and mutation allele. Arrow indicates insertion site. **b-d**, HEK293T cell-based expression of FOXP1, FOXP1mut-p.A339SfsX4, FOXP2, FOXP2-p.R553H, and the alternatively spliced FOXP2-10+ isoform. All transcripts were cloned in a pcDNA4/HisMax expression vector, in frame with its N-terminal Xpress™ tag. **b**, Western blotting of HEK293T transfected whole-cell extracts using an antibody against the N-terminal Xpress™ tag demonstrated the presence of recombinant proteins around the predicted molecular weight for all constructs. The FOXP1mut construct yielded a truncated product of ~45 kDa, approximately half the size of the wild-type protein (FOXP1, ~75 kDa). Levels of the truncated product were similar to those of wild-type FOXP1. The p.R553H variant of FOXP2, an etiological mutation causing severe speech and language deficits in a large multigenerational family¹⁰, was of similar molecular weight to wild-type FOXP2. The 10+ variant represents an alternatively spliced version of FOXP2 encoded by a putative mRNA transcript that contains a polyadenylation site in the intron following exon 10 and thus excludes exons 11-17; as expected this yielded a product of ~50 kDa⁴. Equivalent loading across samples was confirmed using a beta-actin internal loading control. **c**, Confocal images of HEK293T cells transiently transfected with expression constructs followed by immunofluorescence with an antibody to the Xpress™ tag (green). DAPI counterstain (blue) depicts the location of nuclei. Both wild-type FOXP1 and FOXP2 localize predominantly to the nucleus and, in line with previously reported data⁴, are generally excluded from nucleoli. In contrast, the FOXP1mut protein displays predominantly cytoplasmic localization. The FOXP2.R553H protein shows both cytoplasmic and nuclear localization. In some cells it appears to form small aggregates. The FOXP2.10+ isoform is predominantly localized to the cytoplasm and forms cytoplasmic aggregations, which have been suggested to represent aggresomes⁴. Scale bar, 10 μm. **e**, Quantitative RT-PCR results for cDNA prepared

from transiently transfected HEK293T cells. *CNTNAP2* expression levels (y-axis) are the means of three independent cDNA experiments and are normalized with an internal control, *GAPDH*. FOXP1 significantly repressed *CNTNAP2* expression relative to the empty vector (pcDNA4), two-way $p=0.0005$ (**Supplementary Table 8**). Overexpression of FOXP1mut resulted in an approximately three-fold increase in *CNTNAP2* expression relative to the empty vector, two-way $p=0.0056$, suggesting that expression of the mutant FOXP1 protein may lead to amplification of any potentially damaging effects of the *CNTNAP2* H275A allele. Error bars indicate \pm SEM.

Supplementary Table 1 Core descriptive clinical values on ASD probands
Attached file: oroak_supplementary_table1.xlsx

Supplementary Table 2 Exome genotype and Illumina 1M/1MDuo concordance and heterozygous detection rate

Individual	All Overlapping SNP Sites			Heterozygous Overlapping SNP Sites		
	All Concordant	All Sites	# of All Sites	All Detected Het	All Het Sites	# of Het Sites
11048.fa	34209	34293	0.997550521	5951	5971	0.996650477
11048.mo	34070	34167	0.997161003	5913	5932	0.996797033
11048.p1	32761	32854	0.997169294	5556	5575	0.996591928
11307.fa	33528	33606	0.997678986	5701	5713	0.997899527
11307.mo	33411	33498	0.99740283	5909	5925	0.997299578
11307.p1	33425	33521	0.997136124	5647	5669	0.996119245
11580.fa	32976	33095	0.996404291	5745	5782	0.99360083
11580.mo	33488	33590	0.996963382	5776	5794	0.996893338
11666.fa	33389	33491	0.996954406	5647	5677	0.994715519
11666.mo	34267	34363	0.997206297	6018	6041	0.996192683
11666.p1	34488	34562	0.99785892	5901	5922	0.996453901
12325.fa	33994	34074	0.997652169	5726	5747	0.99634592
12325.mo	33900	33987	0.997440198	5897	5916	0.996788371
12325.p1	33914	34013	0.997089348	5684	5710	0.995446585
12499.fa	34742	34821	0.997731254	6090	6114	0.996074583
12499.mo	34744	34820	0.997817346	5933	5947	0.997645872
12499.p1	34650	34724	0.997868909	5862	5875	0.997787234
12575.fa	34021	34096	0.997800328	5892	5909	0.997123033
12575.mo	34771	34838	0.998076813	6051	6066	0.997527201
12575.p1	34504	34577	0.99788877	5923	5932	0.998482805
12647.fa	34619	34684	0.998125937	5916	5922	0.998986829
12647.mo	34751	34836	0.997559995	6161	6170	0.998541329
12647.p1	34019	34122	0.99698142	5926	5946	0.996636394
12681.fa	34540	34623	0.99760275	5961	5984	0.996156417
12681.mo	34744	34816	0.997931985	6279	6289	0.998409922
12681.p1	34115	34192	0.997748011	6092	6108	0.997380485
12817.fa	34532	34600	0.998034682	5979	5984	0.999164439
12817.mo	34772	34831	0.998306107	6172	6180	0.998705502
12817.p1	34313	34391	0.997731965	5944	5959	0.997482799
13095.fa	34392	34473	0.997650335	6095	6117	0.996403466
13095.mo	34123	34203	0.997661024	5958	5973	0.997488699
13095.p1	34452	34529	0.99776999	6109	6123	0.997713539
	AVG		0.997561106	AVG		0.997047046

Supplementary Table 3 Proband sites covered in each trio

Proband	Trio Covered	Total Sites	Total %
11048.p1	13626	14095	0.966725789
11307.p1	12966	13509	0.959804575
11580.p1	13079	13912	0.940123634
11666.p1	13008	14306	0.909268838
12325.p1	13182	13866	0.950670705
12499.p1	14089	14479	0.973064438
12575.p1	13703	14568	0.940623284
12647.p1	13827	14144	0.97758767
12680.p1	13681	14124	0.968634948
12681.p1	14290	14750	0.968813559
12817.p1	13931	14364	0.969855194
12974.p1	13466	13990	0.962544675
13095.p1	13995	14605	0.958233482
13253.p1	13158	13775	0.955208711
13284.p1*	16855	17806	0.946591037
13466.p1	13542	14023	0.965699208
13683.p1	13314	14419	0.923365005
13708.p1	13475	13997	0.962706294
13970.p1	13678	14293	0.956971944
SAGE4022.p1	13772	14538	0.947310497

***13284 Included Additional RefSeq Targets**

Supplementary Table 4 Observed and expected *de novo* coding mutation rates

	Total	Avg	Expected*
All Coding	18	0.9	0.591
Synon	7	0.35	0.14
Missense	9	0.45	0.41
Nonsense	0	NA	0.022
Splice	1	0.05	0.013
Indels	1	0.05	0.035

***Based on a haploid mutation rate of substitution rate of 1.28E-08, deletion rate of 5.80E-10, and insertion rate of 2.00E-10, 22,546,796 coding and 523,843 splice bases covered in an average trio**

Supplementary Table 5 Variant positions of 21 genes with identified *de novo* events from 1000 genomes⁹ pilot data, 20 HapMap, and 20 ASD probands

Attached file: oroak_supplementary_table5.xlsx

Supplementary Table 6 Rare protein disruptive variants intersecting with SFARI gene list

Variants Intersecting Genes Unique to Cases (*de novo* events excluded)

ID	Type	Chromosome:Position	Gene Symbol	Variant	AA Change	GERP Score	Grantham Score
11048.p1	missense	chr16:87874950	ANKRD11	R	P1834L	2.75	98
12680.p1	missense	chr16:87878688	ANKRD11	R	S588L	5.16	145
12325.p1	frameshift	chr3:53817801	CACNA1D	+C			
11307.p1	missense	chr20:44303060	CDH22	R	R167C	3.85	180
11580.p1	missense	chr5:26938483	CDH9	K	K371T	5.19	78
12817.p1	missense	chr7:146449073	CNTNAP2	R	H275R	5.47	29
13253.p1	missense	chr11:522746	HRAS	Y	D154N	3.81	23
13284.p1	missense	chr6:114485886	HS3ST5	W	L90H	5.57	99
13683.p1	missense	chr2:100908186	NPAS2	R	N60S	5.1	46
12325.p1	missense	chr7:107608049	NRCAM	K	K886T	5.08	78
12499.p1	missense	chr15:86481659	NTRK3	Y	R201H	4.23	29
13095.p1	frameshift	chr4:134291098	PCDH10	-C			
12647.p1	missense	chr1:40867593	RIMS3	S	L171V	4.14	32
11580.p1	missense	chr6:166782242	RPS6KA2	Y	R439Q	3.93	43
13683.p1	missense	chr4:53468386	SCFD2	Y	M613V	5.36	21
13466.p1	missense	chr11:70011071	SHANK2	R	V397A	4.52	64
12680.p1	missense	chr15:23201720	UBE3A	Y	D18N*	3.49	23
13970.p1	missense	chr15:23201720	UBE3A	Y	D18N*	3.49	23

*Variant observed in two probands

Variants Intersecting Genes Unique to Controls

ID	Type	Chromosome:Position	Gene Symbol	Variant	AA Change	GERP Score	Grantham Score
1842	missense	chr5:112202615	APC	R	N1142S	5.41	46
1846	missense	chr7:117219988	CTTNBP2	S	K166N	2.45	94
1798	missense	chrX:32276529	DMD	R	S1788L	5.14	145
1843	missense	chrX:12638530	FRMPD4	Y	S521L	5.12	145
1805	missense	chr15:24568644	GABRB3	Y	M80V	4.22	21
1798	missense	chr2:154705212	GALNT13	R	A87T	5.78	58
1804	missense	chr6:102240920	GRIK2	Y	T317M	5.49	81
1838	frameshift	chr6:30018712	HLA-A	+C			
1796	missense	chr7:107389291	LAMB1	S	R642G	4.8	125
1838	missense	chr10:102756781	LZTS2	S	R626G	3.59	125
1804	missense	chr1:160593539	NOS1AP	K	A310S	5.14	99
1804	missense	chr17:7985215	PER1	S	G1257R	3.71	125
1798	missense	chrX:107217677	PSMD10	R	T141I	4.86	89
1840	missense	chr2:166609996	SCN1A	M	L489V	2.84	32
1804	missense	chr16:19102430	SYT17	S	E137D	2.92	45

Variants Intersecting Genes in Both Cases & Controls

ID	Type	Chromosome:Position	Gene Symbol	Variant	AA Change	GERP Score	Grantham Score
13708.p1	missense	chr8:143955163	CYP11B1	R	R24C	1.58	180
1847	missense	chr8:143958147	CYP11B1	Y	A29T	-5.38	58
12575.p1	missense	chr1:97811940	DPYD	Y	I435V	5.34	29
1841	missense	chr1:97753928	DPYD	M	R561L	4.95	102
12575.p1	missense	chr17:17641687	RAI1	Y	P1567L	4.79	98
1796	missense	chr17:17639511	RAI1	Y	R842W	3.6	101
1803	missense	chr17:17639587	RAI1	Y	A867V	4.72	64
12647.p1	missense	chr7:102970438	RELN	Y	R2216Q	5.47	43
1800	missense	chr7:102949739	RELN	R	A2545V	5.37	64
13970.p1	missense	chr16:2078053	TSC2	Y	M1691T	4.3	81
1804	missense	chr16:2078136	TSC2	R	A1719T	4.16	58

Supplementary Table 7 Multiple mutations affecting a single proband

Proband	Type*	Source	Chromosome:Position	Gene Symbol	Variant	AA Change	GERP Score	Grantham Score
11580.p1	missense	inherited	chr5:26938483	CDH9	K	K371T	5.19	78
11580.p1	missense	inherited	chr6:166782242	RPS6KA2	Y	R439Q	3.93	43
12325.p1	frameshift	inherited	chr3:53817801	CACNA1D	+C			
12325.p1	missense	inherited	chr7:107608049	NRCAM	K	K886T	5.08	78
12499.p1	missense	inherited	chr15:86481659	NTRK3	Y	R201H	4.23	29
12499.p1	missense	<i>de novo</i>	chr2:166556317	SCN1A	R	P1894L	5.55	98
12499.p1	missense	<i>de novo</i>	chr6:152865504	SYNE1	Y	Y282C	4.48	194
12499.p1	CNV	inherited	chr15:20300028-20647960	TUBGCP5, CYFIP1, NIPA2, NIPA1	DEL			
12680.p1	missense	inherited	chr15:23201720	UBE3A	Y	D18N	3.49	23
12680.p1	missense	inherited	chr16:87878688	ANKRD11	R	S588L	5.16	145
12817.p1	frameshift	<i>de novo</i>	chr3:71132860	FOXP1	+T	A339SfsX4		
12817.p1	missense	inherited	chr7:146449073	CNTNAP2	R	H275R	5.47	29
13683.p1	missense	inherited	chr2:100908186	NPAS2	R	N60S	5.1	46
13683.p1	missense	inherited	chr4:53468386	SCFD2	Y	M613V	5.36	21

*Includes disruptive *de novo*, SFARI, and copy number rare variants

Supplementary Table 8 *CNTNAP2* expression in HEK293T cell assays

	Mean	SEM
pcDNA4	1.04	±0.03198
FOXP2	0.5592	±0.05054
FOXP1	0.6172	±0.02632
FOXP1mut	3.44	±0.4409

comparison p-value

pcDNA4 vs FOXP2=0.0033
pcDNA4 vs FOXP1=0.0005
pcDNA4 vs FOXP1mut=0.0056
FOXP2 vs FOXP1=ns
FOXP1 vs FOXP1mut=0.0031

Calculated using a two-tailed unpaired *t* test

Supplementary Table 9 Primer sequences for exome capture and qPCR

Adapters

Adapter_PE_Hi	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TC*T
Adapter_PE_Lo	/5Phos/GAT CGG AAG AGC GGT TCA GCA GGA ATG CCG AG

Library Primers

Standard (pre-cap/pre-seq/blocking)

SLXA_Pair_For_Amp	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC *T
SLXA_Pair_Rev_Amp	CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC *T

Barcode

pre-cap

PreCapIndex_Fwd_Amp_Common	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GC
Rev_Amp_w/Barcode	CAA GCA GAA GAC GGC ATA CGA GAT CAA GGT CAC GGT CTC GGC ATT CCT GCT GAA CCG
1/96 barcodes: barcode sequence	CAA GGT CA

blocking

ET-Nbgn-Index-BlockFwd	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T
ET-Nbgn-Index-BlockRev1	CAA GCA GAA GAC GGC ATA CGA GAT
ET-Nbgn-Index-BlockRev1	CAA GCA GAA GAC GGC ATA CGA GAT

pre-seq

Post_Cap_Short_Fwd_Amp	AAT GAT ACG GCG ACC ACC GAG ATC T
Post_Cap_Short_Rev_Amp	CAA GCA GAA GAC GGC ATA CGA GAT

“*” refers to a phosphorothioate bond.

QPCR

CNTNAP2_F	TCC CTC CAC GTC CCA AAA ATG
CNTNAP2_R	TCT TGG CAT AGC CGG GAG AA
GAPDH_F	CAG TCC ATG CCA TCA CTG C
GAPDH_R	TTC GTT GTC ATA CCA GGA AAT G

Supplementary References

1. Fischbach, G.D. & Lord, C. The Simons Simplex Collection: a resource for identification of autism genetic risk factors. *Neuron* **68**, 192-5 (2010).
2. Lord, C. et al. The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. *J Autism Dev Disord* **30**, 205-23 (2000).
3. Lord, C., Rutter, M. & Le Couteur, A. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord* **24**, 659-85 (1994).
4. Vernes, S.C. et al. Functional genetic analysis of mutations implicated in a human speech and language disorder. *Hum Mol Genet* **15**, 3154-67 (2006).

5. MacDermot, K.D. et al. Identification of FOXP2 truncation as a novel cause of developmental speech and language deficits. *Am J Hum Genet* **76**, 1074-80 (2005).
6. Vernes, S.C. et al. A functional genetic link between distinct developmental language disorders. *N Engl J Med* **359**, 2337-45 (2008).
7. Bakkaloglu, B. et al. Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. *Am J Hum Genet* **82**, 165-73 (2008).
8. Vissers, L.E. et al. A de novo paradigm for mental retardation. *Nat Genet* (2010).
9. Durbin, R.M. et al. A map of human genome variation from population-scale sequencing. *Nature* **467**, 1061-73 (2010).
10. Lai, C.S., Fisher, S.E., Hurst, J.A., Vargha-Khadem, F. & Monaco, A.P. A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature* **413**, 519-23 (2001).